The Vascular Disrupting Agent STA-9584 Exhibits Potent Antitumor Activity by Selectively Targeting Microvasculature at Both the Center and Periphery of Tumors

Kevin P. Foley, Dan Zhou, Chris Borella, Yaming Wu, Mei Zhang, Jun Jiang, Hao Li, Jim Sang, Tim Korbut, Josephine Ye, Xuemei Zhang, James Barsoum, and Andrew J. Sonderfan

Synta Pharmaceuticals Corp., Lexington, Massachusetts
Received May 25, 2012; accepted July 25, 2012

ABSTRACT
Vascular disrupting agents (VDAs) are an emerging class of therapeutics targeting the existing vascular network of solid tumors. However, their clinical progression has been hampered because of limited single-agent efficacy, primarily caused by the persistence of surviving cells at the well perfused “viable rim” of tumors, which allows rapid tumor regrowth to occur. In addition, off-target adverse events, including cardiovascular toxicities, underscore a need for compounds with improved safety profiles. Here, we characterize the mechanism of action, antitumor efficacy, and cardiovascular safety profile of STA-9584, a novel tubulin-binding VDA. In vitro, STA-9584 induced significant tumor regressions in prostate and breast xenograft models in vivo and, in an aggressive syngeneic model, demonstrated superior tumor growth inhibition and a positive therapeutic index relative to combretastatin A-4 phosphate (CA4P). It is noteworthy that histological analysis revealed that STA-9584 disrupted microvasculature at both the center and periphery of tumors. Compared with CA4P, STA-9584 induced a 73% increase in central necrotic area, 77% decrease in microvasculature, and 7-fold increase in tumor cell apoptosis in the remaining viable rim 24 h post-treatment. Ultrasound imaging confirmed that STA-9584 rapidly and efficiently blocked blood flow in highly perfused tumor regions. Moreover, cardiovascular effects were evaluated in the Langendorff assay and telemetered dogs, and cardiovascular toxicity was not predicted to be dose-limiting. This bioactivity profile distinguishes STA-9584 from the combretastatin class of therapeutic agents and identifies the compound as a promising new therapeutic VDA candidate.

Introduction
Tumor vasculature is essential for the supply of oxygen and nutrients that promote solid tumor growth and the spread of metastatic disease. Hence targeting this network represents an intuitively attractive and clinically validated approach for the treatment of human malignancies (Tozer et al., 2005; McKeage and Baguley, 2010). First-generation therapeutics focused on the use of angiogenesis inhibitors, which prevent the neovascularization process and/or promote vascular normalization within tumors (Grothey and Galanis, 2009). This resulted in the successful clinical development of a number of new anticancer drugs, including bevacizumab
Vascular disrupting agents (VDAs) represent a relatively new group of agents that exhibit selective activity against established tumor vascular networks, causing rapid collapse and shutdown of tumor blood flow, ultimately culminating in extensive necrosis of the tumor mass (Kanthou and Tozer, 2009). The susceptibility of tumor vessels, but not normal vasculature, to damage by VDAs has been ascribed to structural differences, including their immature pericyte-deficient nature, high intrinsic permeability, and chaotic organization (Dvorak et al., 1988; Eberhard et al., 2000; Hashizume et al., 2000).

VDAs are categorized into two types, biological (ligand-directed) and small molecule. Biological VDAs typically combine an endothelium-targeted molecule with a toxin or procoagulant. Small-molecule VDAs can be further subdivided into two classes based on their mechanisms of action. One class contains the flavonoid VDAs, such as 5,6-dimethylxanthone-4 acetic acid (ASA404), that exert antivascular effects through induction of local cytokine production (Gaya and Rusnnone-4 acetic acid (ASA404), that exert antivascular effects through induction of local cytokine production (Gaya and Rusnnone-4 acetic acid (ASA404). However, the largest group of small-molecule VDAs is made up of tubulin binding agents, which target tumor endothelial cells by exploiting their dependence on the tubulin cytoskeleton to maintain cell shape and vascular integrity. All are structurally related to colchicine and include combretastatin A-4 (CA4), its prodrug CA4 phosphate (CA4P), the CA4P analog [3-methoxy-2- phosphonatoxy-6-[(Z)-2-(3,4,5-trimethoxyphenyl)ethenyl]phenyl phosphate (Oxi4503) (combretastatin A1 phosphate), and (2S)-2-amino-3-hydroxy-N-[2-methoxy-5-[(Z)-2-(3,4,5-trimethoxyphenyl)ethenyl]phenyl] propanamide (AVE8062). methyl-[5-[[4-[[2-(aminopropanoyl)amino]phenyl]sulfanyl]-1H-benzimidazol-2-yl]carbamate monohydrochloride (MN-029). N-acetylcycloliginin-O-phosphate (ZD6126), (3Z,6Z)-3-[[5-tert-butyl-1H-imidazol-4-yl]methylen]-6-(phenylmethylene)-2,5-piperazinedinedione (NPI-2358), and N-ethyl-N-[2-methoxy-4-5-methyl-4-[[1S]-1-(3-pyridinyl)butyl]amino]-2-pyrimidinyl]phenyl]urea (CYT97). The comparative effects of STA-9122 and CA4 treatment on cell lines that represent tumors of diverse hematologic and solid tumor origin were all obtained from the American Type Culture Collection (Manassas, VA) and maintained according to the suppliers’ instructions. Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza Hopkinton, Inc. (Hopkinton, MA). STA-9122 and its prodrug STA-9584 were synthesized by Synda Pharmaceuticals Corp. from 3,4,5-trimethoxybenzaldehyde with structures determined by H NMR and mass spectroscopy and confirmed by combustion analysis. The combretastatins (Z)-2-methoxy-3-(4,5-trimethoxybenzyl)aniline (AVE8063), CA4, and the prodrug CA4P were synthesized according to published procedures (for CA4: Pettit et al., 1985; for the H NMR and mass spectra of the synthesized materials conformed to published data.

In Vitro HUVEC Assays. To examine microtubule depolymerization, HUVECs were exposed to 5 nM STA-9122, CA4, or AVE8063 for 24 h. Cells were fixed with 3% formaldehyde followed by cold methanol treatment to preserve both microtubule structures and depolymerized tubulin within the cytoplasm. Cells were immunostained with an antibubulin antibody (DM1A; Sigma-Aldrich, St. Louis, MO), and microtubule structures were visualized by using fluorescence microscopy.

The comparative effects of STA-9122 and CA4 treatment on HUVEC migration were determined by using a wound healing assay. HUVECs were grown to confluence on 24-well plates before scraping to generate linear wounds. The cells were then cultured in the presence of 1 nM STA-9122, 1 or 5 nM CA4, or DMSO (control). Time-lapse imaging was performed by using the IncuCyte Live-Cell Imaging System (Eissen Bioscience, Ann Arbor, MI) where the cells were kept at 37°C and 5% CO2 during the imaging process, and plates were photographed at 24, 48, and 72 h. Cells migrating into wound regions were quantified by manual counting.

Disruption of vascular structures in vitro was investigated by using a capillary tube assay. HUVECs were first seeded in Matrigel-coated 24-well plates in growth factor-rich EGM-2 medium (Lonza Hopkinton, Inc.) overnight to permit formation of capillary tube structures. Cells were exposed to increasing concentrations of STA-9122 or CA4 (1–100 nM) for 1 h and then cultured for an additional
48 h. Disruption of capillary tubes was assessed by using light microscopy at 10× magnification.

**Cell Viability Assays.** HUVECs were treated with 10-fold serial dilutions of STA-9122 or CA4 (1000–0.1 nM) or vehicle (DMSO; ≤ 0.5%) for 1 h, and viability was assessed at 48 h. Tumor cell lines were seeded overnight and then dosed with graded concentrations of STA-9122 for 72 h. For primary cell assays, primary cultures of rat PBMCs and hepatocytes were used. In brief, whole blood was collected from an adult male Sprague Dawley rat, and PBMCs were isolated by using Ficoll density centrifugation. Freshly isolated PBMCs were cultured in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum and seeded into 96-well plates at 1 × 10⁵ cells/well. For hepatocyte isolation, livers were perfused and harvested from anesthetized adult male Sprague Dawley rats followed by a collagenase digestion. Hepatocytes were isolated by low-speed centrifugation and suspended in serum-free HepatoStim Medium (BD Biosciences Discovery Labware, Bedford, MA) plus 2% bovine serum albumin. This method yielded >85% viability as determined by trypan blue exclusion. Hepatocytes were seeded into collagen-coated 96-well plates at 1.5 × 10⁵ cells/well and allowed to attach for 2 h. Primary cell cultures were then treated with STA-9122 at concentrations of 2, 10, 25, and 100 μM for 20 h. At the end of the respective incubations, WST-8 reagent (Cell Counting Kit-8; Dojindo Molecular Technologies, Inc., Rockville, MD) was added to the cells, and absorbance was detected at a wavelength of 450 nm. Optical density readings were used to generate a standard curve from which the IC₅₀ values of the compounds were determined by using XLI-Fit software (ID Business Solutions, Guildford, UK).

**In Vivo Xenograft Tumor Models.** Female BALB/c, immunodeficient BALB/c nude, and CB-17/Scid-Prkdc-scid (SCID) mice (Charles River Laboratories Inc., Wilmington, MA) at 7 to 12 weeks of age were maintained in a pathogen-free environment, and all in vivo procedures were approved by the University Institutional Animal Care and Use Committee. PC-3 and MDA-MB231 tumor cells (5 × 10⁶) were subcutaneously implanted into nude and SCID mice, respectively, and EMT6 cells (1 × 10⁵) were subcutaneously implanted into BALB/c mice. Animals bearing established tumors (100–200 mm³; >500 mm³ for MDA-MB-231 xenografts) were randomized into treatment groups of 8 to 10 and intravenously dosed via the tail vein with either vehicle or STA-9584 formulated in 5% dextrose in water. Studies were conducted at 4.5 mg/kg STA-9584 once a week; in the syngeneic EMT6 model, animals were also treated with CA4P at 100 mg/kg on the same dosing schedule. Tumor growth inhibition was determined as described previously (Proia et al., 2011). Statistical analyses were performed by using a Kruskal-Wallis one-way analysis of variance on ranks followed by the Tukey test.

**Histological Assessment of EMT6 Xenografts.** EMT6 tumor-bearing mice (n = 10) were administered a single dose of STA-9584 (4.5 mg/kg), CA4P (100 mg/kg), or vehicle for 24 h. Paraffin-embedded sections from the maximal radial regions of each tumor were subject to terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining by using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore Corporation, Billerica, MA) and counterstained with 2% methyl green. Sections were also stained with an anti-CD31 antibody (BD Pharmingen, San Diego CA)/hematoxylin. Images were acquired by using a Nikon (Tokyo, Japan) E800 microscope and Leica (Wetzlar, Germany) DC camera linked to Image-Pro plus software (Media Cybernetics, Inc., Bethesda, MD). Under low magnification, the dense TUNEL-positive area correlated with apoptotic/necrotic changes in corresponding hematoxylin and eosin-stained sections. Thus, the percentage of apoptotic/necrotic area was determined by the average TUNEL-positive area/average total area at 20× magnification. To determine the proportion of apoptotic cells in viable tumor areas, image analysis was performed by using five randomly selected areas per slide at 200× magnification. For quantitative evaluation of CD31-positive cells, a total of six randomly selected areas were collected from within the viable zones of two independent sections per tumor sample (200× magnification). Data are expressed as the average CD31 positivity per tumor sample as a percentage of the control. Statistical significance was determined by one-way analysis of variance.

**High-Resolution In Vivo Imaging of Blood Flow.** Evaluation of tumor perfusion was performed in mice bearing EMT6 xenograft tumors and carried out under anesthesia (2% isofluorane in oxygen). Tumors were imaged by using a Vevo 770 Micro-Ultrasound platform (VisualSonics, Inc., Toronto, Ontario, Canada), the RMV 706 scanner, Contrast Analysis Software, and MicroMarker untargeted ultrasound contrast agent. Using the RMV 706 scanner, images were collected at a high spatial resolution (lateral and axial resolution of 100 and 40 μm, respectively; focal length, 6 mm; transmit power, 50%; mechanical index, 0.14; dynamic range, 52 dB). Tumor perfusion was measured by using Contrast Mode Software in conjunction with MicroMarker untargeted ultrasound contrast agent. Lyophilized contrast agent was first reconstituted in 0.7 ml of a sterile sodium chloride solution (0.9% w/v). A predose baseline was collected by injecting a 50-μl bolus intravenous injection of contrast agent and capturing the resulting perfusion characteristics. STA-9584 was immediately dosed at 4.5 mg/kg at t = 0 h, and at 4 h contrast agent imaging was repeated and the resultant perfusion characteristics were captured.

**Langendorff Assay.** In brief, hearts from male New Zealand white rabbits (Clerco Farm LLC, Cincinnati, OH) were used to measure the physiological variables of PQ interval, QRS complex, RR interval, corrected QT interval (QTc), developed left ventricular pressure (LVP, d), and coronary blood flow after perfusion with escalating doses of STA-9584 or STA-9122 (10⁻⁵ to 10⁻³ M) (see Supplementary Materials and Methods for complete details). Mean values were calculated for each concentration, and mean values (± S.E.M.) were plotted against concentration for all parameters assessed, for both STA9584/STA-9122-exposed and vehicle-treated hearts.

**Cardiovascular Study.** Three male Beagle dogs (Covance Inc., Kalamazoo, MI) previously instrumented with systemic blood pressure and ECG telemetry devices (Data Sciences International, St. Paul, MN) were used in the experiment. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). The study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of CorDynamics (Chicago, IL), where the studies were performed. STA-9584 (0.1, 0.25, or 0.5 mg/kg) was administered intravenously into a cephalic or saphenous vein through an in-dwelling catheter for 1 h. Dogs were returned to their home run at the end of the dosing. Each dosing was conducted with 1-week intervals according to a dose-escalating paradigm. Systemic blood pressure, heart rate, and ECG measurements were recorded continuously for 24 h after dosing via telemetry. The QT interval was corrected for heart rate by using Van De Water’s formula (Van de Water et al., 1989). A 2-ml blood sample for determination of STA-9584 plasma concentrations was collected immediately before and just before the end of infusion.

**Results**

STA-9122 Exhibits Potent Microtubule Inhibition Activity in Endothelial Cells and Cytotoxic Effects on Tumor Cells In Vitro. STA-9122 (Fig. 1A) is a novel small-molecule VDA that targets the colchicine-binding (MT) site of tubulin (Supplemental Fig. S1). Accordingly, STA-9122 treatment of HUVECs resulted in complete disruption of microtubule structures and more effectively than equivalent doses of the combretastatins AVE8063 (the active component of AVE8062) or CA4 (the active metabolite of CA4P) (Fig. 1B). STA-9122 inhibited HUVEC migration in vitro at a 5-fold lower dose than CA4 (Fig. 1C). STA-9584 was more potent than CA4 in HUVEC cytotoxicity assays (IC₅₀ values of 30 versus 175 nM, respectively) and at disrupting endo-
thelial cell capillary tube formation (IC$_{50}$ 4 versus 10 nM) (Table 1). In a primary cell cytotoxicity screening, STA-9122 had little effect on cell viability (Table 1) but was broadly cytotoxic with low nanomolar potency in a panel of 20 human cancer cell lines (Fig. 1D).

**STA-9584 Exhibits Potent Single-Agent Efficacy in Tumor Xenografts In Vivo.** Because of the limited solubility of STA-9122, for in vivo studies we generated a water-soluble phenylalanine ester prodrug, STA-9584 (Fig. 1A), and evaluated its activity in a series of xenograft models. Initially, BALB/c nude mice bearing established PC-3 human prostate cancer xenografts were intravenously dosed with STA-9584 on a weekly schedule at its maximal tolerated dose (MTD) of 4.5 mg/kg (Fig. 2A). Significant tumor regression (40%) was induced by STA-9584 compared with the control group. This regimen was well tolerated with minimal loss of body weight observed during the course of treatment (Fig. 2B).

We then examined the antitumor effects of STA-9584 in mice bearing large established tumors. To do this, SCID mice were implanted with MDA-MB-231 human breast carcinoma cells, and tumors were allowed to reach >500 mm$^3$ before treatment (Fig. 2C). Weekly dosing of STA-9584 was also highly efficacious in this model, which resulted in 60% tumor regression. To date, STA-9584 has shown substantial antitumor efficacy in each of six xenograft models of human and murine tumors of diverse origins (Table 2). STA-9584 showed dose-dependent effects in regimens below its MTD and, as is the case with other VDAs, tumor growth could become reinitiated upon removal of the compound (data not shown).

To directly compare the in vivo efficacies of STA-9584 and CA4P, we next conducted a study in wild-type, immunocompetent BALB/c mice subcutaneously implanted with the highly aggressive, syngeneic (BALB/c-derived) mouse EMT6 breast carcinoma cell line. In nontumor-bearing BALB/c mice, the MTDs were determined to be 4.5 mg/kg for STA-9584 and 100 mg/kg for CA4P when dosing once a week for 2 weeks (data not shown). Weekly administration of STA-9584 (4.5 mg/kg) was significantly more efficacious than CA4P dosed at its MTD of 100 mg/kg (T/C values of 11 versus 53%, respectively) (Fig. 2D). In fact, the 100 mg/kg CA4P dose failed to meet the National Cancer Institute’s %T/C ≤42 standard for significant efficacy. It is noteworthy that even a 200 mg/kg dose (which caused cardiac toxicities) also failed to meet this standard and was still much less efficacious than 4.5 mg/kg STA-9584 (%T/C = 43%; data not shown).

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Endothelial Cell Capillary Tube Disruption</th>
<th>Endothelial Cell Cytotoxicity</th>
<th>Primary Cell Cytotoxicity Panel</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA4</td>
<td>10</td>
<td>175</td>
<td>N.D.</td>
</tr>
<tr>
<td>STA-9122</td>
<td>4</td>
<td>30</td>
<td>47,500–84,100</td>
</tr>
</tbody>
</table>

N.D., not determined.
STA-9584 Rapidly and Completely Blocks Tumor Blood Flow in a Syngeneic Breast Cancer Model.

The vascular disrupting activity of STA-9584 was examined by using the EMT6 model. First, the effects of STA-9584 and CA4P on tumor blood flow were compared by using the standard Evan's blue dye extravasation assay (as described in Supplemental Materials and Methods) on EMT6 tumors ex vivo, where the compounds inhibited blood flow by 61 and 43\% at 4 h after drug treatment, respectively (Supplemental Fig. S2). Next, we used a high-resolution contrast ultrasound imaging technique to examine tumor blood flow in vivo. Animals were intravenously injected with a microbubble contrast agent and immediately imaged to obtain a predose measurement of tumor blood flow. STA-9584 was then dosed at its MTD, and 4 h later contrast agent was again injected and animals were imaged for a second time. Figure 3A shows representative images before and after treatment with STA-9584, showing that blood flow was completely blocked in highly perfused tumor subregions within 4 h of drug administration. As shown in Fig. 3B, blood flow was monitored by changes in the contrast intensity within the tumors, which was completely abrogated after STA-9584 treatment. Similar results were observed in three of three animals.

STA-9584 Antitumor Effects Are Not Confined to the Central Region of Tumors. In light of this potent antivascular activity, and to further account for STA-9584’s superior efficacy in vivo, EMT6 tumor-bearing animals were treated with single doses of STA-9584 (4.5 mg/kg) or CA4P (100 mg/kg) for 24 h, at which point tumors were isolated for histological analysis. It is noteworthy that a single dose of STA-9584 completely arrested tumor growth over this period, as measured by changes in tumor volumes, in contrast to only a slight delay with CA4P (Supplemental Fig. S3). Tumor sections were analyzed by using methyl green staining to detect viable tissue and TUNEL staining to identify regions of apoptosis/necrosis (Fig. 4A). As expected, tumors from CA4P-treated animals showed central areas of necrosis; however, in every tumor large regions of viable cells persisted, particularly at the periphery (representing the viable rim). In contrast, STA-9584-exposed xenografts displayed far more extensive cellular destruction throughout the entire tumor.
including areas of fibrosis and dramatically reduced viable rim or surviving tumor zones. In fact, many tumor sections from STA-9584-treated animals showed no detectable viable rim tissue. Overall, the core necrotic/apoptotic region as a percentage of total tumor area increased by 73% after treatment with STA-9584 relative to CA4P (Fig. 4B).

This finding suggested that STA-9584 blocked blood flow by specifically disrupting tumor microvasculature at both the center and periphery of tumors. To confirm this, anti-CD31 immunohistochemistry was performed and endothelial cells were counted in the remaining periphery/viable rim of tumors (excluding the central necrotic area). Tumors from STA-9584-treated animals, but not CA4P, contained large numbers of disrupted microvessels with decreased, patchy CD31 expression, loss of integrity, and thrombosis (Fig. 4C). In total, a 77% decrease in CD31+/H11001 endothelial cells in the viable rim was observed after treatment with STA-9584 relative to CA4P (Fig. 4D). Concomitant changes in levels of apoptosis were seen when TUNEL-positive cells were counted in the same region (Fig. 4C). There was little change in the number of apoptotic cells in the viable rim after treatment with CA4P relative to vehicle alone. However, a 7-fold increase in apoptotic cells was observed after treatment with STA-9584 relative to CA4P (Fig. 4E). Taken together, these data suggest that the increased in vivo efficacy of STA-9584 relative to CA4P (Fig. 2D) can be attributed, at least in part, to increased microvasculature destruction and consequent apoptosis in peripheral regions of tumors.

STA-9584 Has a Favorable Cardiovascular Safety Profile. Cardiotoxicity has emerged as an important dose-limiting toxicity for a number of VDAs that have entered clinical development, including CA4P (Subbiah et al., 2011). For this reason, the cardiovascular effects of escalating doses of STA-9584 and STA-9122 on electrophysiology (RR, PQ, QRS, and QTc) and mechanical properties (LVPdev and coronary blood flow) were evaluated in isolated rabbit hearts by using the Langendorff assay. It is noteworthy that STA-9122 had no significant effect on any parameter over the concentration range tested (10<sup>-8</sup> to 10<sup>-5</sup> M) (Supplemental Fig. S4). For the STA-9584 prodrug, no significant effects on electrophysiological parameters were seen at concentrations 10<sup>-6</sup> M (Fig. 5A). At the highest 10<sup>-5</sup> M dose, PQ interval, QRS duration, and QTc increased compared with baseline, and slight decreases in LVPdev and blood flow were observed (Fig. 5A). However, it is important to note that this concentration is 50-fold higher than the efficacious dose C<sub>max</sub> value (2 10<sup>-7</sup> M). Expected physiological changes on these same parameters were observed by using increasing concentrations of quinidine (10<sup>-8</sup> to 10<sup>-4</sup> M) as a positive control (data not shown).

In a rising-dose tolerance study of STA-9584 in Beagle dogs, the MTD was determined to be 0.5 mg/kg with the major dose-limiting toxicities involving transient clinical signs typical of many tubulin-binding cytotoxic agents (emesis, diarrhea, and bloody stool) and select hematological changes (data not shown). It is noteworthy that no treat-
ment-related cardiac effects were observed. To extend this finding, we performed a cardiovascular function study in dogs surgically instrumented with blood pressure/ECG telemetry devices. Dogs \((n = 3)\) were given rising doses of 0.1, 0.25, and 0.5 mg/kg STA-9584 (1 h infusion) with 1 week between doses, and blood pressure/ECG measurements were monitored for 24 h postdosing. As shown in Fig. 5B, no ECG (QTc) effects were observed at any dose or any heart rate or pressure changes seen with 0.1 and 0.25 mg/kg dosing. Cardiac effects of STA-9584 administration at the MTD (0.5 mg/kg) were restricted to transient elevations in mean arterial pressure (driven primarily by increases in diastolic blood pressure) and heart rate between 1 and 4 h (Fig. 5B). No notable changes in electrocardiographic intervals (PR, QRS, and QTc) or arrhythmogenesis related to STA-9584 were seen at any doses (data not shown). Overall, these data show that cardiovascular toxicity is not predicted to be dose-limiting for STA-9584.

Discussion

Functional disruption of the tumor vasculature is an established approach for the development of novel antineoplastic therapies. VDAs differ in mechanism to antiangiogenic agents, which prevent neovascularization, by compromising the preexisting network to rapidly shut down blood flow, thus preventing the supply of oxygen and nutrients. Here, we report the preclinical characterization of STA-9584, a promising new investigational VDA of the tubulin binding class. Consistent with other microtubulin-destabilizing agents, STA-9584 targeted endothelial cells of established tumor blood vessels, rapidly blocking blood flow to elicit tumor necrosis. Unlike most other VDAs, however, STA-9584 displayed potent single-agent antitumor activity in vivo, including tumor regressions in xenograft models of prostate and breast cancer, even in large established tumors. It is noteworthy that we found that the superior bioactivity exhibited by this compound was related to its capacity to specifically disrupt tumor microvasculature and induce cell death at both the center and periphery of tumors (Fig. 6).

CA4P is the prototypical member of the combretastatin class and was the first VDA to enter clinical trials (Dowlati et al., 2002). Here, we report that STA-9584 was substantially more efficacious than CA4P by using a highly aggressive syngeneic breast cancer xenograft model. Moreover, morphological examination revealed significantly increased cellular destruction throughout tumors exposed to STA-9584, leading to a dramatic reduction in surviving tumor zones. This superior therapeutic index of STA-9584 suggests an activity profile similar to that of the second-generation CA4P analog Oxi4503. Compared with CA4P, Oxi4503 has been shown to exert more potent antivascular activity and tumor growth delays; treatment also results in the persistence of a relatively smaller viable rim (Hill et al., 2002b; Holwell et al., 2002; Hua et al., 2003). The enhanced efficacy of Oxi4503 has been ascribed to the in vivo generation of reactive quinone species that may be cytotoxic to tumor cells through free radical formation and elevation of oxidative stress (Folkes et al., 2007; Rice et al., 2011). Here, we showed that the active STA-9122 moiety, which itself is stable and does not generate reactive quinones, was also potently cytotoxic to a panel of...
tumor cell lines of diverse hematologic and solid tumor origins. Thus it is reasonable to suggest that the direct induction of cancer cell death, in addition to vascular disruption, contributes to the robust single-agent activity of STA-9584.

To date, the clinical experience with VDAs has failed to deliver on the therapeutic potential of this class of antivasular agents. Tumor regrowth from the viable rim under- scores one of the major shortcomings of VDA monotherapy, contributing to both limited efficacy and treatment resistance. Accordingly, investigation of VDAs as a complementary approach to standard of care chemotherapeutics or other targeted agents is a primary focus of ongoing clinical evaluations (Eichholz et al., 2010; McKeage and Baguley, 2010).

One of the most advanced agents, ASA404, showed potential clinical benefit in early phase I and II trials in non–small-cell lung cancer (NSCLC) in combination with paclitaxel/carbo-platin (McKeage et al., 2008, 2009; Hida et al., 2011). These promising findings then prompted examination of the combination as part of first- or second-line therapy for advanced NSCLC in two large-scale randomized phase III clinical tri-
als; however, both were halted when no overall survival benefit could be demonstrated (Lara et al., 2011; Lorusso et al., 2011). Overall, intense efforts at developing VDAs with increased efficacy have been underway in recent years (Siemann, 2011) and, in light of the observations we are presenting here, STA-9584 exhibits a preclinical activity profile of significant therapeutic potential.

Another factor contributing to the lack of clinical progression is the prevalence of toxicity profiles marked by off-target VDA effects on the cardiovascular system. The most common effects include hypertension, ischemia, arrhythmias, atrial fibrillation, and myocardial infarction (for review see Subbiah et al., 2011). Although some signs/symptoms (e.g., low-grade hypertension) may be transient and manageable for many VDAs, in some cases these toxicities can be significantly dose-limiting and have resulted in termination of the development of the drug, as seen for (5S)-5-acetylaminolino-9,10,11-trimethoxy-6,7-dihydro-5H-dibenzo[a,e]cyclohepten-3yl dihydrogenphosphate (ZD6216) (LoRusso et al., 2008). With respect to the combretastatins, adverse cardiovascular events have been reported for CA4P when administered either alone or in combination with other cytotoxic agents (Dowlati et al., 2002; Rustin et al., 2003, 2010). As an initial step in evaluating the suitability of STA-9584 for development as a clinical candidate, a comprehensive assessment of the cardiovascular safety profile of the compound was performed. The evaluation revealed no effects of STA-9122 on any electrophysiological or mechanical parameter in the Langendorff assay, and only minor alterations in QRS duration, QTc, LVP, and blood flow were observed for STA-9584 at the highest concentration examined. It is noteworthy that this concentration was 50-fold higher than the efficacious dose $C_{\text{max}}$ value, which is unlikely to be approached in a therapeutic setting. In addition, no treatment-related cardiac effects were observed in Beagle dogs, either as part of a rising-dose tolerance study or telemetered cardiovascular function study. Taken together, these findings show that cardiac toxicity is not likely to be dose-limiting for STA-9584. This represents an important discriminating feature of the compound, because both hypertension (as a common adverse drug reaction) and atrial fibrillation (as a dose-limiting toxicity) have recently been reported in a phase I evaluation of Oxi4503 (Patterson et al., 2012).

In summary, we have developed and characterized a novel small-molecule VDA with distinct bioactivity and potent antitumor efficacy in preclinical models of human cancer. Furthermore, the favorable cardiovascular profile exhibited by STA-9584 predicts for a superior therapeutic index compared with other classes of tubulin-binding agents. Taken together, the data presented here identify STA-9584 as a promising new therapeutic VDA candidate.


Address correspondence to: Andrew Sonderfan, Synta Pharmaceuticals Corp., 125 Hartwell Avenue, Lexington, MA 02421. E-mail: asonderfan@syntapharma.com
The vascular disrupting agent STA-9584 exhibits potent antitumor activity by selectively targeting microvasculature at both the center and periphery of tumors

Kevin P. Foley, Dan Zhou, Chris Borella, Yaming Wu, Mei Zhang, Jun Jiang, Hao Li, Jim Sang, Tim Korbut, Josephine Ye, Xuemei Zhang, James Barsoum, and Andrew J. Sonderfan.

Journal of Pharmacology and Experimental Therapeutics

Supplemental Materials and Methods

Competitive colchicine binding assay

The effects of STA-9122 on the binding of colchicine to tubulin were analyzed by scintillation proximity assays performed using a colchicine site competitive assay kit (Cytoskeleton, Denver, CO) according to manufacturers’ instructions. Briefly, assays were conducted using biotin-labeled tubulin and streptavidin-coated scintillation proximity assay (SPA) beads. The reaction system contained [\(^{3}H\)]colchicine (Perkin-Elmer, Waltham, MA), equimolar concentrations of unlabeled STA-9122 or colchicine, and biotin-labeled tubulin attached to streptavidin SPA beads. Reactions were performed in G-PEM buffer (80 mmol/L PIPES, 2 mmol/L MgCl\(_2\), 1 mmol/L EGTA, and 0.1 mmol/L GTP) and incubated at 37°C for 90 min prior to scintillation counting using a Wallach scintillation counter.

Evans Blue dye uptake assay

BALB/c mice bearing EMT6 xenografts were dosed with CA4P (100 mg/kg), STA-9584 (4.5 mg/kg) or vehicle and Evan’s Blue dye was i.v. injected via the tail vein at 1 h. Mice were sacrificed and dye extracted from tumors at 4 h and spectrophotometrically quantitated as a measurement of tumor blood volume, perfusion and plasma extravasation.
**Langendorff assay**

Hearts from male New Zealand white rabbits were surgically removed, placed on the perfusion apparatus, and control measurements of all physiological variables (PQ, QRS, RR, QT, and dLVP/dt) collected for 15 min during the perfusion with Krebs solution (equilibration period). Four hearts were then perfused with the vehicle for 15 min followed by perfusion with escalating doses of STA-9584 or STA-9122 (10^{-8} – 10^{-5} M), with measurements taken the last 60 sec of each 15 min epoch. Data was collected for the entire period of exposure. The heart from one animal was perfused with vehicle solution (negative control) and another heart was perfused with escalating concentrations of the positive control (quinidine). For each 15 min epoch the last 60 sec period of collection was analyzed and presented. Coronary blood flow was measured by collecting the effluent over the final 2 min of each 15 min epoch of perfusion. Data was acquired on the EMKA IOX Data Acquisition System. Measurements for all physiological parameters (PQ, QRS, RR, QT, LVP_{dev}) were made manually using EMKA ECG Auto software [QTc(Fridericia^2) was calculated] from beats manifesting sinus rhythm. All physiological parameters were analyzed for each collection interval. Mean values for each parameter were calculated for each concentration, and mean values (±SEM) were plotted against concentration for all parameters assessed, both for STA-9854/STA-9122-exposed and vehicle-treated hearts.
The vascular disrupting agent STA-9584 exhibits potent antitumor activity by selectively targeting microvasculature at both the center and periphery of tumors

Kevin P. Foley, Dan Zhou, Chris Borella, Yaming Wu, Mei Zhang, Jun Jiang, Hao Li, Jim Sang, Tim Korbut, Josephine Ye, Xuemei Zhang, James Barsoum, and Andrew J. Sonderfan.

Synta Pharmaceuticals Corp., Lexington, MA

Figure S1. STA-9122 binds to the colchicine-binding site of tubulin. Tubulin was incubated with [3H]colchicine in the presence or absence of unlabeled STA-9122 or colchicine at 37°C for 90 min and the amount of radioligand bound determined using scintillation proximity assay beads. Competition binding curves with [3H]colchicine are shown.
The vascular disrupting agent STA-9584 exhibits potent antitumor activity by selectively targeting microvasculature at both the center and periphery of tumors

Kevin P. Foley, Dan Zhou, Chris Borella, Yaming Wu, Mei Zhang, Jun Jiang, Hao Li, Jim Sang, Tim Korbut, Josephine Ye, Xuemei Zhang, James Barsoum, and Andrew J. Sonderfan.

*Synta Pharmaceuticals Corp., Lexington, MA*

**Figure S2.** Evans Blue dye (EBD) extravasation assay. BALB/c mice bearing EMT6 xenografts (n = 8/group) were dosed with CA4P (100 mg/kg), STA-9584 (4.5 mg/kg) or vehicle and Evan’s Blue dye was i.v. injected via the tail vein at 1 h. Dye was extracted from tumors at 4 h and spectrophotometrically quantitated as a measurement of tumor blood volume and perfusion. Dye extravasation was decreased by 61% and 43% for STA-9584 and CA4P, respectively. *, p < 0.05 vs. vehicle control.
The vascular disrupting agent STA-9584 exhibits potent antitumor activity by selectively targeting microvasculature at both the center and periphery of tumors.

Kevin P. Foley, Dan Zhou, Chris Borella, Yaming Wu, Mei Zhang, Jun Jiang, Hao Li, Jim Sang, Tim Korbut, Josephine Ye, Xuemei Zhang, James Barsoum, and Andrew J. Sonderfan.

Synta Pharmaceuticals Corp., Lexington, MA

Figure S3. Single MTD dose of STA-9584 blocked tumor growth. BALB/c mice bearing EMT6 xenografts (n = 10/group) were administered a single dose of CA4P (100 mg/kg), STA-9584 (4.5 mg/kg) or vehicle for 24 h. Tumor volume measurements were made by caliper and changes in volume at 24 h are presented. *, p < 0.003 vs. vehicle control.
The vascular disrupting agent STA-9584 exhibits potent antitumor activity by selectively targeting microvasculature at both the center and periphery of tumors

Kevin P. Foley, Dan Zhou, Chris Borella, Yaming Wu, Mei Zhang, Jun Jiang, Hao Li, Jim Sang, Tim Korbut, Josephine Ye, Xuemei Zhang, James Barsoum, and Andrew J. Sonderfan.

Synth Pharmaceuticals Corp., Lexington, MA

**Figure S4.** Effects of escalating concentrations of STA-9122 and vehicle on RR, PQ, QRS and QTc(F) intervals, left ventricular developed pressure (LVPdev) and coronary blood flow in male rabbit hearts. STA-9112 values presented as the mean (± SEM); n=4. Vehicle values presented from one animal.